

REVIEW ARTICLE

Membrane protein secretases

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A diverse range of membrane proteins of Type I or Type II topology also occur as a circulating, soluble form. These soluble forms are often derived from the membrane form by proteolysis by a group of enzymes referred to collectively as 'secretases' or 'shedases'. The cleavage generally occurs close to the extracellular face of the membrane, releasing physiologically active protein. This secretion process also provides a mechanism for down-regulating the protein at the cell surface. Examples of such post-translational proteolysis are seen in the Alzheimer's amyloid precursor protein, the vasoregulatory enzyme angiotensin converting enzyme, transforming growth factor- α , the tumour necrosis factor ligand and receptor superfamilies, certain cytokine

receptors, and others. Since the proteins concerned are involved in pathophysiological processes such as neurodegeneration, apoptosis, oncogenesis and inflammation, the secretases could provide novel therapeutic targets. Recent characterization of these individual secretases has revealed common features, particularly sensitivity to certain metalloprotease inhibitors and up-regulation of activity by phorbol esters. It is therefore likely that a closely related family of metallosecretases controls the surface expression of multiple integral membrane proteins. Current knowledge of the various secretases are compared in this Review, and strategies for cell-free assays of such proteases are outlined as a prelude to their ultimate purification and cloning.

INTRODUCTION

The mechanism of protein secretion in eukaryotic cells has long fascinated cell biologists. It is now clear, however, that there is no single biosynthetic mechanism common to all secretory proteins. Secretion of proteins can occur through either the regulated or constitutive pathways and, in some cell types, this secretion can be polarized to distinct cellular domains. The sorting of proteins between the regulated and constitutive pathways takes place in the *trans*-Golgi network, and the mechanisms underlying these trafficking events are now being dissected at the molecular level and have been recently reviewed [1]. An increasing number of secreted proteins are now recognized as being derived from integral plasma membrane proteins and, in this case, the secretory event involves their selective post-translational hydrolysis from the cell surface. This secretion (often called 'shedding' or 'solubilization') involves either a protease or a phospholipase, depending on the type of membrane anchor on the protein (see below). Proteins secreted in this fashion include some membrane receptors and receptor ligands, ectoenzymes, cell adhesion molecules, and others. We have coined the generic term 'membrane protein secretases' for the proteases that generate soluble isoforms of membrane proteins and first characterized this process in relation to the secretion of angiotensin converting enzyme (ACE; peptidyl dipeptidase A; EC 3.4.15.1) [2]. The present Review will focus on the characteristics of such membrane protein secretases (sometimes referred to in the literature as 'membrane protein convertases' or 'shedases') and their potential as novel therapeutic targets. For earlier reviews on this topic, see [3,4].

MEMBRANE PROTEIN TOPOLOGY

Integral transmembrane proteins

Distinctions are made between integral transmembrane proteins on the basis of their orientation and the number of times the protein spans the lipid bilayer (Figure 1). Type I proteins (e.g. ACE) are synthesized with a cleavable N-terminal signal peptide which is removed early in biosynthesis, but they are retained in the membrane by a hydrophobic sequence of amino acids close to the C-terminus, together with so-called 'stop-transfer' (charged) amino acids (Figure 1a). In the case of the Type II cell-surface proteins (e.g. endopeptidase-24.11; neprilysin; EC 3.4.24.11) the membrane anchor is the uncleaved signal peptide and the protein is oriented with a short, hydrophilic, cytoplasmic domain and with the bulk of the protein, including the C-terminus, facing the extracellular space (Figure 1b). Some single membrane-spanning proteins, especially those involved in intracellular signalling processes (e.g. receptor-tyrosine kinases, or membrane guanylyl cyclases) exhibit a 'dumb-bell' structure in which the transmembrane segment is towards the middle of the protein sequence such that a significant proportion of the protein is exposed at both membrane surfaces. Finally, a wide range of multiple membrane-spanning proteins exist (Type III), ranging from two transmembrane segments in the case of subunit *c* of the F_0 -ATPase complex through to 24 in the case of the sodium channel in neurons.

The endogenous proteolytic release of integral transmembrane proteins is limited to those of Type I and Type II structure in which the cleavage site is generally located close to the membrane

Abbreviations used: ACE, angiotensin converting enzyme; AP, alkaline phosphatase; APP, β -amyloid precursor protein; β A4, β -amyloid peptide; CHO, Chinese-hamster ovary; 3,4-DCI, 3,4-dichloroisocoumarin; DFP, di-isopropyl fluorophosphate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; FasL, Fas ligand; GPI, glycosyl-phosphatidylinositol; IL6R, interleukin 6 receptor; KL, Kit ligand; PKC, protein kinase C; sAPP α , secreted APP cleaved at the α -secretase site; TGF- α , transforming growth factor- α ; TIMP, tissue inhibitor of metalloproteases; TNF- α , tumour necrosis factor- α ; TNFR, tumour necrosis factor receptor.

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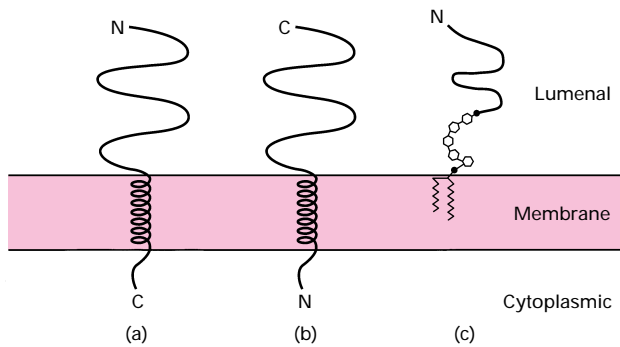


Figure 1 Integral membrane protein topology

(a) Type I integral membrane protein with a cleaved N-terminal signal sequence and a C-terminal membrane anchoring sequence; (b) Type II integral membrane protein with the uncleaved signal sequence doubling as the membrane anchor; (c) GPI-anchored membrane protein.

surface such that the bulk of the protein is released into the extracellular milieu, often in a fully functional form. In the majority of cases it is likely that the cleavage occurs at a single, unique site defined by the specificity of the secretase and the topology of the protein substrate. A model for this process is provided by the known ease of solubilization of certain hydrolases of the renal and intestinal brush borders by treatment with proteases, especially papain or trypsin. This procedure has often been used to facilitate purification of membrane proteins (see [5]

for discussion). Negative-staining electron microscopy has revealed that these brush-border hydrolases appear as ‘knobs’ separated from the surface of the plasma membrane by a short stalk that can vary in length from 2 to 9 nm depending upon the protein (Figure 2a). ‘Short-stalked’ proteins, such as endopeptidase-24.11 (stalk length 2 nm [6]), cannot be released by papain (the shortest dimension of a papain molecule is 3.6 nm), whereas those with a substantially longer stalk (e.g. aminopeptidase N; EC 3.4.11.2) are readily cleaved (Figure 2a) [7]. One can envisage a similar mechanism operating for the membrane protein secretases in which cleavage of a protein substrate depends upon access to a stalk region close to the membrane surface (Figure 2b). Thus only certain cell-surface proteins will be susceptible to release, as is the case *in vivo*.

Type III proteins with multiple transmembrane segments cannot, of course, be released from the membrane by limited proteolysis, but specific proteolytic cleavage can, in some cases, modify their membrane activities. This is best exemplified by the recently identified family of protease-activated receptors typified by the thrombin receptor [8,9]. Here, thrombin cleaves its receptor within the extracellular N-terminus, forming an N-terminal tethered ligand that activates the receptor. In contrast, the ligand-induced cleavage of the V_2 vasopressin receptor by a plasma-membrane metalloprotease appears to terminate the action of the receptor [10]. Another example is the thyrotropin receptor, whose large glycosylated ectodomain is cleaved by a protease that is blocked by the metalloprotease inhibitor BB2116 (see Figure 4 below) [11].

Lipid-anchored proteins

A separate group of integral proteins are anchored to the

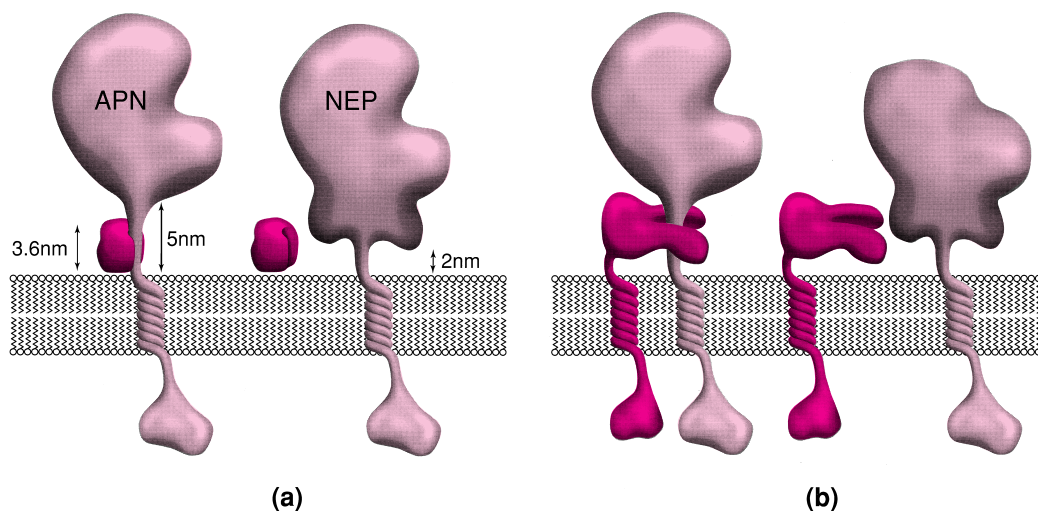


Figure 2 Mechanism of action of secretases

(a) Aminopeptidase N (APN; stalk length 5 nm) is released from the brush-border membrane by papain (shortest dimension 3.6 nm), whereas endopeptidase-24.11 (NEP; stalk length 2 nm) is not released. (b) Action of a membrane-bound secretase on two different membrane proteins; only the membrane protein with the longer membrane-proximal stalk region is susceptible to release by that particular secretase.

Table 1 Integral membrane proteins with soluble isoforms generated by proteolysis

This Table is modified from [3,4]. Although numerous other membrane proteins are found as soluble forms in serum and other body fluids their mode of production is unknown. Abbreviations used: ANF, atrial natriuretic factor; CSF, colony-stimulating factor; GH, growth hormone; IL, interleukin; LAM, leucocyte adhesion molecule; LAR, leucocyte common antigen-related protein; MHC, major histocompatibility complex; NCAM, neural-cell adhesion molecule; NGF, nerve growth factor; PDGF, platelet-derived growth factor; VCAM-1, vascular cell-adhesion molecule; VSV, vesicular-stomatitis virus.

Type	Protein	Topology	Reference(s)	
Cell-adhesion molecules	CD14	GPI	[129]	
	CD8 (Leu-2)	Type I	[107,130]	
	L-selectin (gp100MEL-14, LAM-1, Leu-8)	Type I	[108,109,131]	
	VCAM-1 (CD106)	Type I	[132]	
	ICAM-3 (CD50)	Type I	[133]	
	NG2 proteoglycan	Type I	[134]	
Leucocyte antigens	Class I MHC	Type I	[135]	
	Tac (IL2 receptor)	Type I	[136]	
	CD16-I (Fc γ RIII-1)	GPI	[137]	
	CD16-II (Fc γ RIII-2)	Type I	[105]	
	CD23 (IgE receptor)	Type II	[138,139]	
	CD32 (Fc γ RII)	Type I	[140]	
	CD43	Type I	[101,104]	
	CD44	Type I	[102,141]	
Receptor ligands	TGF- α	Type I	[72,142]	
	TNF- α	Type II	[78,80]	
	KL-1 (Kit ligand)	Type I	[72,95]	
	KL-2 (Kit ligand)	Type I	[72,95]	
	CSF-1	Type I	[143]	
	CD40	Type II	[144]	
	Fas	Type II	[83]	
	TNFR-I (p55/60)	Type I	[88]	
	TNFR-II (p75/80)	Type I	[145]	
	NGF receptor	Type I	[146]	
Receptors	CSF-1 receptor	Type I	[147]	
	Transferrin receptor	Type II	[148]	
	Poly-Ig receptor	Type I	[149]	
	Folate receptor	GPI (Type I)	[118,119]	
	ANF receptor	Type I	[150]	
	IL-1 receptor (II)	Type I	[151]	
	IL-6 receptor	Type I	[89,100,152]	
	LAR-protein tyrosine phosphatase	Type I	[153]	
	TGF- β receptor (β -glycan)	Type I	[154,155]	
	PDGF receptor	Type I	[156]	
	Syndecan	Type I	[157]	
	CD27	Type I	[158]	
	CD30	Type I	[92]	
	Ectoenzymes	ACE	Type I	[50,61]
		Sialyltransferase	Type II	[159]
	Viral membrane proteins	Leukaemogenic glycoprotein (gp55)	Type I	[160]
		VSV glycoprotein	Type I	[161]
Miscellaneous proteins	Amyloid precursor protein	Type I	[162]	
	Glycoprotein Ib	Type I	[163]	

membrane via a covalently attached glycosyl-phosphatidylinositol (GPI) moiety (for a review, see [12,13]). A diverse group of membrane proteins are now known to be anchored in this fashion in eukaryotic cells, including cell-adhesion molecules, differentiation antigens, tumour markers, certain receptors and also some ectoenzymes [14]. The alkyl or acyl chains of the GPI structure provide the sole attachment of the protein to the external face of the plasma membrane (Figure 1c). A number of mammalian GPI-anchored proteins can also be detected in a circulating, hydrophilic form in serum. This could occur by proteolysis (as in the case of the GPI-anchored folate receptor; see below), but, additionally, the GPI anchor provides a mechanism for release of such proteins from the cell surface through the action of phospholipases C or D. The ability of bacterial phosphatidylinositol-specific phospholipase C to release GPI-anchored proteins from the cell surface was first recognized for

alkaline phosphatase (AP) [15,16]. There is good evidence that the endogenous serum GPI-phospholipase D is involved in the release of AP [17] and the basic fibroblast growth factor–heparan sulphate proteoglycan complexes from bone-marrow cultures [18]. Also, a soluble form of 5'-nucleotidase appears to be derived from the membrane-bound form through the action of a GPI-phospholipase C [19].

MEMBRANE PROTEINS WITH SOLUBLE ISOFORMS THAT ARE GENERATED BY PROTEOLYSIS

Numerous integral plasma-membrane proteins are now known to be released from the lipid bilayer by proteolysis (Table 1). These include cell adhesion molecules and leucocyte antigens, receptors and receptor ligands, ectoenzymes and viral membrane proteins. The biological function of the proteolytic release of

Table 2 Properties of membrane protein secretases

See the text for details.

Substrate	Class of protease	Activated by Phorbol esters?	Site of cleavage* (... P ₁ ↓ P ₁ ' ...)	Distance from membrane†
APP				
α-Secretase	Metallo-	Yes	VHHQK ↓ LVFFA	12
β-Secretase	?Serine	No	SEVKM ↓ DAEFR	28
γ-Secretase	?Cysteine/serine	n.d.‡	VGGVV ↓ IATVI	Within membrane
ACE	Metallo-	Yes	EAGQR ↓ LATAM	93
			PNSA R ↓ SEGPL	27
			AQQAR ↓ VG QWL	3
TGF-α	Metallo- (serine)	Yes	ADLLA ↓ VVAAS	9
TNF-α	Metallo- (serine)	Yes	PLAQA ↓ VRSSS	20
Fas ligand	Metallo-	Yes	n.d.	
TNFR-I	Metallo-	Yes	PQIEN ↓ VKGTE	10
TNFR-II	Metallo-	n.d.	APGAV ↓ HLPQP	43
CD30	Metallo-	Yes	n.d.	
KL-1	Serine	Yes	PPVAA ↓ A ↓ SSLRN	25/24
KL-2	Serine	Yes	n.d.	
IL6R	Metallo-	Yes	SLAVQ ↓ DSSSV	1
CD43	Metallo-/serine	Yes	n.d.	
CD44	Metallo-/serine	Yes	n.d.	
CD16-I	Metallo-/serine	Yes	n.d.	
CD16-II	Metallo-	Yes	n.d.	
L-selectin	Metallo-	Yes	QKLDK ↓ SFSMI	11
Folate receptor	Metallo-	n.d.	EEVA ↓ R ↓ F ↓ YAAA	Approx. 11

* Nomenclature of Schechter and Berger [164].

† Number of amino acids from the predicted transmembrane domain to the secretase cleavage site.

‡ n.d., not determined.

membrane proteins varies. In some cases it may be a process for rapidly down-regulating the protein from the surface of the cell, in others it may be to generate a soluble form of the protein that has properties either identical with, or subtly different from, those of the membrane-bound form. However, despite widespread observation of this phenomenon for a number of years, it is only in the last couple of years that significant progress has been made in characterizing the membrane secretases responsible (Table 2). The properties of these secretases are presented below.

PROPERTIES OF MEMBRANE PROTEIN SECRETASES

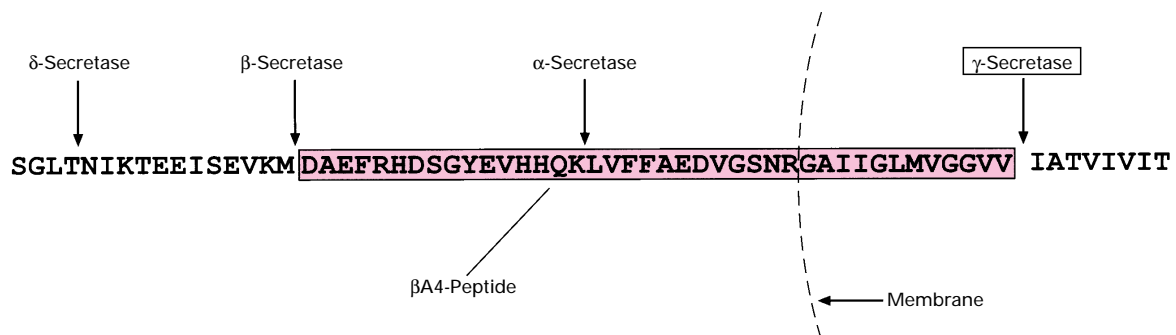
β-Amyloid precursor protein secretases

β-Amyloid precursor protein (APP) is a Type I integral membrane protein that is ubiquitously expressed on cells. APP has been

intensively studied over the last decade since the 4 kDa peptide (βA4) that formed the amyloid filaments in Alzheimer's-disease patients was isolated [20,21]. The deposition of βA4 is currently believed to be the central pathological event in the development of Alzheimer's disease [22]. APP can be cleaved by at least three secretases, termed α-, β- and γ-secretase, as shown in Figure 3.

α-Secretase

The α-secretase cleavage site precludes the formation of the amyloidogenic βA4 peptide, releasing an extracellular portion of APP termed 'sAPPα' (secreted APP cleaved at the α-secretase site) [23]. A range of studies using cell lines transfected with different APP isoform cDNA constructs have resulted in a consensus viewpoint that α-secretase cleavage occurs pre-

**Figure 3** Cleavage sites for the APP secretases

dominantly between Lys¹⁶ and Leu¹⁷ (amino acid numbering from the N-terminus of the β A4 peptide). In fact this has been difficult to demonstrate unequivocally, owing to the potential for amino- and/or carboxy-peptidase removal of the Lys residue after the primary cleavage event [23–25]. The studies that have explored the amino acid specificity of α -secretase have revealed some unusual features. It was reported that amino acid substitutions around the cleavage site (see Figure 3), and a large deletion mutant that removed the α -secretase cleavage site entirely, did not prevent APP cleavage [26]. With the deletion mutant, the cleavage was shown to occur at a Glu–Val bond that was 12 amino acids distal from the transmembrane domain: the same number of residues distant as the normal Lys¹⁶–Leu¹⁷ bond. From that study came the notion that α -secretase cleavage was more dependent on distance from the membrane than on amino acid sequence. By progressively deleting the extracellular juxtamembrane amino acid sequence from 35 to five amino acids from the transmembrane domain, it was shown that 11 amino acids of the natural sequence comprised the minimum required to sustain APP cleavage [27]. With just five amino acids of the juxtamembrane sequence remaining, or its complete deletion, cleavage was prevented.

The amino acid specificity of α -secretase was investigated in an elegant study where the residues around the Lys¹⁶–Leu¹⁷ cleavage site were systematically mutated [28]. These studies revealed that Lys¹⁶ \rightarrow Val (P₁), Val¹⁸ \rightarrow Gly (P₂') and Phe¹⁹ \rightarrow Pro (P₃') inhibited α -secretase cleavage significantly, with the latter two substitutions predicted to perturb any α -helical secondary structure that may be present. Insertion of three amino acids C-terminal to the α -secretase cleavage site increased the size of the sAPP α fragment that was released into the medium. These findings are consistent with α -secretase cleaving at a particular distance from the membrane, as well as having amino acid sequence specificity. However, other workers have demonstrated, in different cell culture systems, that alternative cleavage sites are available [29]. Using sensitive radiosequencing techniques, it was shown that while 60% of α -secretase cleavage occurred at Lys¹⁶–Leu¹⁷, about 40% could be ascribed to the Phe¹⁹–Phe²⁰ position. These workers also made a series of substitutions and deletion constructs around the favoured Lys¹⁶–Leu¹⁷ cleavage site, none of which was able to prevent the release of sAPP α . Using two deletion mutants C-terminal to the cleavage site, α -secretase cleavage was governed by amino acid sequence and did not cleave at positions that would indicate a distance-from-membrane specificity. Some, but not all, of the amino acid substitutions around the cleavage site that are predicted to perturb the α -helical nature of the region were also effective in preventing cleavage, and this may indicate that an α -helical structure is an important recognition element for α -secretase. An added complication to the interpretation of these studies is the difficulty in distinguishing between a single protease having a 'relaxed' amino acid specificity and a family of α -secretases having quite specific, but different, activities. One interpretation of the data on the site of cleavage by α -secretase is that the enzyme cleaves predominantly on the N-terminal side of hydrophobic residues, but that the enzyme has an extended substrate-binding site such that residues distant from the scissile bond can have a marked influence on the site of cleavage [30].

Thus far there has been only a single reported attempt to establish a cell-free assay to characterize α -secretase (see below) [31]. In that study a reporter cDNA construct (AP–APP) was used in which the N-terminal domain of AP was linked to the C-terminal 105 amino acids of APP (which is therefore truncated five amino acids N-terminal to the Met⁻¹–Asp¹ β -secretase cleavage point) (Figure 3). Using specific anti-

bodies to the C-terminal region of sAPP α that would detect α -secretase cleaved AP–APP, together with C-terminal sequencing of the released AP–APP reporter protein, it was demonstrated that, in transfected cells, α -secretase cleavage occurred at the prototypic Lys¹⁶–Leu¹⁷ cleavage site. On incubation of membrane preparations from transfected cells, it was shown that an α -secretase activity was able to release AP–APP from the membrane in a time- and temperature-dependent manner. The α -secretase activity was not affected by a range of class-specific protease inhibitors, with the exception of the zinc-chelating agent, 1,10-phenanthroline. Intriguingly, this agent was markedly more effective if the membranes were first washed using a detergent, implying that the active site of the protease was, in some way, inaccessible. Thus the activity revealed by these experiments would appear to be an integral membrane metalloprotease. Additional support comes from a recent report that details how cholesterol supplementation to cultured cells is able to decrease α -secretase activity, presumably by reducing membrane fluidity and thereby reducing interaction of α -secretase with APP [32]. It is not difficult to envisage that membrane anchorage of the active site of α -secretase might limit the access of the protease to certain juxtamembrane regions of a similarly membrane-anchored APP substrate (see Figure 2b). The cell regulation of α -secretase has also received considerable attention, particularly the effects of agents able to activate protein kinase C (PKC). Thus phorbol ester treatment of cells increases sAPP α release [33]. In addition, it has recently been shown that protein kinase A also stimulates the production of sAPP α [34].

β - and γ -secretases

β -Secretase and γ -secretase will be considered together, because many of the studies in this area focus on the production of β A4 peptide, which is the product of both activities (Figure 3). Despite intense research, there have been no data that define the β - or γ -secretase at the biochemical level. Initial experiments showed that, on the basis of the lack of a precursor–product relationship in the production of p3 (the product of α - and γ -secretase) and β A4, the two peptides were generated independently from different APP molecules. This was supported by data that showed that, whereas NH₄Cl treatment was able to depress β A4 production, p3 was relatively unaffected [35]. The precise intracellular locations for the production of β A4 have yet to be identified, although on the basis of a range of observations it now seems quite unlikely that β A4 is produced within lysosomes, since leupeptin, an inhibitor of lysosomal proteases, fails to prevent β A4 production [35,36]. Also, I cells that have defective lysosomal function are still able to produce β A4 [35], and β A4 cannot be detected within purified lysosomes [37]. However, agents such as monensin and brefeldin-A, which interfere with Golgi function, or NH₄Cl, which is acidotropic and will neutralize early endosomal compartments, are able to prevent β A4 secretion [35].

The cell-biological parameters of β A4 secretion have been explored by using a range of APP cDNA constructs. If the cytoplasmic domain of APP is removed, β A4 production proceeds as long as the transmembrane domain can still function to anchor the APP molecule to the membrane. If membrane attachment of APP is compromised, then β A4 production is abolished [38]. Intact APP that escapes α -secretase cleavage and reaches the cell surface can be re-internalized and cleaved to produce β A4 [39]. Although this may only represent a small proportion of the total APP that is processed to β A4, a precursor–product relationship was demonstrated between C-terminal fragments and β A4. An extensive study explored the

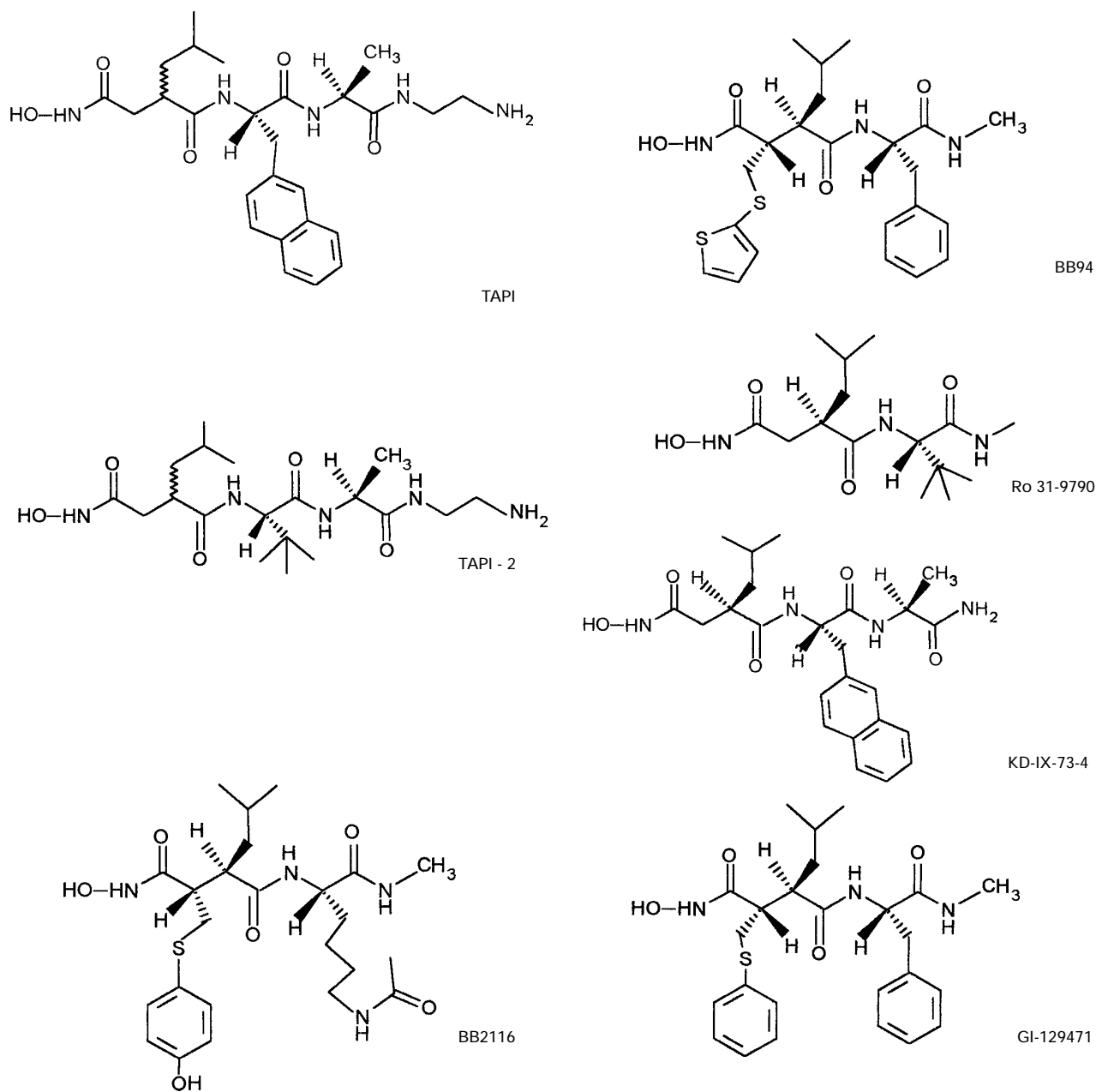


Figure 4 Structures of metallosecretase inhibitors

The structures of the various hydroxamic acid-based zinc-metalloprotease inhibitors that have been shown to inhibit one or more membrane protein secretases are shown.

cleavage site amino acid specificity of β -secretase [38]. Using amino acid deletion and insertion constructs in the β A4 sequence between the α - and β -secretase cleavage sites it was shown that β A4 production was unaffected in human kidney 293 cells. This demonstrated that β -secretase did not cleave with a distance-from-membrane specificity. Mutations to the amino acid sequence around the cleavage site revealed that β -secretase was intolerant to most changes. At the P_1 position, β A4 production was maintained by substituting the wild-type Met⁻¹ (numbering from the N-terminus of β A4; Figure 3) with other bulky hydrophobic residues such as Phe and Tyr. β A4 secretion was markedly up-regulated by substituting the P_1 Met⁻¹ with Leu, as

occurs with the Swedish double mutation [40]. Similarly, the only substitution to the P_1' residue that was able to support β -secretase cleavage was Asp¹ \rightarrow Glu. Thus it would appear from the extensive mutation studies performed at the β -secretase cleavage site that this protease is highly sequence-specific. It is also clear that APP needs to be membrane-associated for β -secretase cleavage to occur, although there is no evidence as yet that β -secretase itself is a membrane-associated protease. Recently, it has been reported that the broad-spectrum serine-protease inhibitor 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride blocks the constitutive production of β A4 in several cell lines, probably by inhibition of β -secretase [41].

It seems likely that β -secretase cleaves before γ -secretase, probably within an endosomal compartment. γ -Secretase has the unusual property of cleaving in a region of the protein that is predicted to reside within the transmembrane region of the APP molecule. Thus γ -secretase must either be able to cleave within the membrane environment or the membrane must first be degraded. Another possibility is that there is a cleavage event within the cytosolic domain of APP, followed by a rapid 'nibbling' of the transmembrane region up to the C-terminus of the β A4 peptide. To focus on some of the properties of γ -secretase, cells were transfected with N-terminally truncated APP constructs equivalent to β -secretase-cleaved APP (named SPA4CT) [42]. Although SPA4CT expression produced an increase in β A4 levels, p3 was not detected. This strongly suggests that β -secretase cleaved C-terminal APP is not a substrate for α -secretase. The calpain inhibitor MDL 28170 was able to block completely β A4 and p3 production from APP-transfected Chinese-hamster ovary (CHO) cells [43]. Treatment with the inhibitor also resulted in the accumulation of C-terminal APP with N-termini equivalent to α - and β -secretase-cleaved APP. In addition, a C-terminal fragment equivalent to cleavage at a Thr⁻¹³-Asn⁻¹² site was identified. This cleavage event has also been described in primary cultures of rat hippocampal neurons transfected with APP using a Semliki-Forest-virus vector, and has been termed δ -cleavage [44]. Using a variety of treatments it was demonstrated that the most likely site of action of the protease inhibited by MDL 28170 was a post-Golgi pre-lysosomal compartment. As both p3 and β A4 production were inhibited, it seems likely that the compound was acting as a γ -secretase inhibitor, although there is no evidence so far that calpain itself is γ -secretase. The substrate specificity of γ -secretase has not been explored as intensively as for β -secretase, and the mechanistic class of γ -secretase has yet to be unequivocally identified. However, MDL 28170 possesses an aldehyde 'warhead' which is consistent with γ -secretase being either a cysteine or a serine protease. The possibility still remains, however, that MDL 28170 was affecting an event upstream of γ -secretase cleavage. It is noteworthy that β A4 is rarely detected within cells, which is consistent with the location of γ -secretase being close to, or at, the plasma membrane, or within a very rapidly recycling endocytic vesicle.

ACE secretase

ACE plays a key role in the control of blood pressure and fluid and electrolyte homeostasis. In mammals, ACE exists as two distinct isoenzymes derived from a single gene by transcription from one of two alternative promoters [45–47]. Although ACE exists primarily as a membrane-bound enzyme, a soluble form is present under normal conditions in blood plasma, amniotic fluid, seminal plasma and other body fluids (reviewed in [46,48]). In certain diseases such as sarcoidosis, diabetes mellitus, Gaucher's disease, leprosy and hyperthyroidism, the levels of soluble ACE in plasma are known to be altered [48,49].

In 1987, while studying the mode of membrane anchorage of porcine kidney ACE, we observed that the enzyme could be selectively released in a time- and temperature-dependent manner from the membrane in a soluble, hydrophilic form by a post-translational proteolytic cleavage event probably involving a secretase [2]. Further characterization of ACE secretase revealed that it was not affected by inhibitors of serine, thiol or aspartic proteases, but was sensitive to inhibition by EDTA and 1,10-phenanthroline, although EGTA was without effect [50]. The inhibition by EDTA was reversed by Mg²⁺ and to some extent by Zn²⁺ and Mn²⁺. Recently the metalloprotease nature of ACE

Table 3 Effect of hydroxamic acid-based zinc-metalloprotease inhibitors on the release of membrane proteins

Abbreviations used: n.d., not determined; n.i., no inhibition; IC₅₀, concentration causing 50% inhibition.

Inhibitor	Protein released	IC ₅₀ (mM)	Reference
TAPI	TNF- α	50–100	[78]
	IL6R	5–10	[89]
	TNFR I (p60)	5–10	[89]
	TNFR II (p80)	25–50	[90]
	CSF-1	n.i.	[89]
TAPI-2	TGF- α	10	[73]
	APP	10	[73]
	L-selectin	10	[73]
	L-selectin	1.0	[114]
	IL6R	10	[73]
BB2116	ACE	1–20	[51]
	ACE	18.3	[52]
	TNF- α	0.23	[79]
	ACE	3.5	[52]
BB2275	CD30	n.d.	[92]
	TNF- α	0.5	[74]
	TNFR-I (p55)	0.8–2.0	[74]
BB94	TNFR-II (p75)	0.8	[74]
	ACE	1.6	[52]
	Fas ligand	2.0	[84]
Ro 31-9790	L-selectin	2.0	[115]
KD-IX-73-4	L-selectin	4.5	[112]
GI-129471	TNF- α	0.18	[80]

secretase has been confirmed with the identification of TAPI-2, BB94 (batimastat) and BB2116 (Figure 4 and Table 3), as inhibitors of this activity ([51]; S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work). Studies with several compounds structurally related to BB94 revealed a different structure–activity relationship towards ACE secretase as compared with collagenase (S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work). Thus, while BB94 and many related compounds are relatively non-selective inhibitors of matrix metalloproteases [53], these results imply that marked differences exist between the recognition features essential for the inhibition of matrix metalloproteases and ACE secretase, and reinforce the earlier suggestion [50] that the latter is a unique, albeit related, zinc-metalloenzyme.

Subcellular fractionation, detergent solubilization ([50]; S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work) as well as pulse–chase and surface-labelling experiments [51,54,55] have established that ACE secretase co-localizes with its substrate, ACE, in the plasma membrane. Interestingly ACE secretase was readily solubilized from the membrane by either Triton X-100 or CHAPS, whereas octyl glucoside was ineffective (S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work), a pattern similar to that observed for the APP α -secretase [31]. In addition, the secretase was released in an active form from the membrane with trypsin (S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work), implying that it may also have a protease-susceptible stalk region. Transfection of the full-length cDNA of either the somatic or testicular isoenzymes results not only in the expression of the membrane-bound form of ACE on the surface of the cells, but also in a secreted form as a result of secretase action [54,56–58]. The release of recombinant ACE from trans-

ected cells and the release of ACE from cultured endothelial cells was up-regulated by phorbol ester treatment [54,55,59,60].

The site of cleavage by the secretase within ACE has been determined for both the somatic and testicular isoenzymes. When the human somatic isoenzyme was expressed in CHO cells, the secretase cleavage site was identified at Arg¹¹³⁷-Leu¹¹³⁸ (some 93 residues N-terminal to the hydrophobic membrane-spanning domain), a result confirmed by analysis of human plasma ACE [61]. Mutation of Arg¹¹³⁷ to Gln had no effect on the observed processing, suggesting that either the secretase could accommodate this change or use an alternative cleavage site. When the rabbit testicular isoenzyme was expressed in a mouse epithelial cell line, the secretase cleavage site was identified at Arg⁶⁶³-Ser⁶⁶⁴ (Arg¹²⁰³-Ser¹²⁰⁴ in human somatic ACE numbering) [54]. Recently, a mutant of human somatic ACE lacking the N-terminal domain was found to be secreted 10-fold more quickly than the wild-type in CHO cells, with cleavage occurring at Arg¹²²⁷-Val¹²²⁸ [55]. Corvol and colleagues suggest that this shift in the position of the secretase cleavage site nearer to the plasma membrane may be due to the N-terminal domain of ACE acting as a conformational inhibitor of the proteolysis by interacting either with the stalk region of ACE or with the secretase itself. Two earlier studies reinforce this concept, where domain-specific antibodies indicated that the two catalytic sites in somatic ACE appear to be in close proximity [62] and radiation inactivation analysis of porcine lung and testicular ACE demonstrated that the two domains in the somatic isoenzyme are structurally tightly linked [63]. The difference in the site of cleavage between the rabbit testicular isoenzyme and the N-terminally deleted mutant of human somatic ACE may simply be due to the presence in human ACE of a potential N-linked glycosylation site eight residues N-terminal to Arg¹²⁰³, which is absent in rabbit ACE, causing additional conformational restrictions on the secretase gaining access to this region of the protein.

Transforming growth factor- α (TGF- α) secretase

TGF- α is a member of the epidermal growth factor family of ligands. Members of this family are typified by having six conserved cysteine residues that form three intrachain disulphide bonds. TGF- α is a 50-amino-acid soluble ligand that is released from a 160-amino-acid membrane protein precursor [64] by cleavage at two very similar amino acid sequences [65]. A clear precursor-product relationship was shown between membrane-associated and soluble, mature TGF- α by using metabolic labelling and pulse-chase experiments [66]. Cleavage at the N-terminus of TGF- α occurs quite rapidly, with cleavage at the C-terminus close to the transmembrane domain occurring more slowly unless cells are stimulated with phorbol esters. The N-terminal cleavage could be mediated by exogenous pancreatic elastase, although the C-terminal cleavage was resistant. The C-terminal cleavage site is at Ala⁸⁹-Val⁹⁰, which is nine amino acids upstream of the transmembrane region and seven amino acids downstream of one of the conserved cysteine residues involved in forming one of the disulphide bonds [65].

The amino acid specificity of TGF- α secretase that acts at the C-terminal cleavage site was investigated by using site-directed mutagenesis [67]. By replacing both the P₁ Ala and P₁' Val residues with Ser and Thr respectively (mutant 1), release of active TGF- α was markedly inhibited. Mutant 2 also incorporated a P₃' Ala \rightarrow Pro mutation, which effectively abolished TGF- α processing. The lack of total inhibition with mutant 1 demonstrated either that a protease lacking sequence specificity was responsible or that the mutant protein was rendered susceptible to a different secretase with a lower overall activity. The

cytoplasmic domain of the protein is highly conserved between species, suggesting an important functional role. A series of cytoplasmic domain deletion and substitution mutants were expressed in CHO and 3T3 cells, where it was shown that the C-terminal Val residue was crucial for controlling the rate of cleavage of TGF- α from the surface of cells [68]. The Val could be replaced with Leu or Ile without preventing cleavage. Kit ligand (KL) also possesses a C-terminal Val, and a TGF- α -KL chimaera (extracellular domain TGF- α -intracellular domain KL) was also shown to release mature TGF- α , implying that a similar mechanism also exists for Kit ligand. However, subsequent studies on Kit ligand have shown this not to be the case [69].

Some information on the essential components of TGF- α processing was derived using streptolysin O-permeabilized cells [70]. Here, cells lacking most of their cytosolic components were still able to release metabolically labelled cell-surface TGF- α after PMA stimulation in the presence of an ATP-generating system. That study also presented some data suggesting that G-proteins were implicated in TGF- α processing, as guanosine 5'-[thio]triphosphate was able to stimulate cleavage. A novel approach to investigate the biochemical events underlying secretase cleavage of cell-associated proteins involved using ethyl methanesulphonate (methylsulphonic acid ethyl ester) to mutagenize cells, followed by a selection process for cells that were unable to cleave cell-surface TGF- α [71]. These cells were shown to process TGF- α normally to the cell surface, although they were unable to cleave TGF- α in response to phorbol ester treatment. APP was similarly affected, indicating a common pathway for the cleavage of these two cell-surface proteins. In fact, this defect in the cells profoundly affected their ability to release a wide range of cell-surface proteins upon phorbol ester stimulation.

TGF- α secretase was inhibited by some serine-protease inhibitors [e.g. 3,4-dichloroisocoumarin (3,4-DCI) and di-isopropyl fluorophosphate (DFP)], but not others (e.g. α_1 -antitrypsin) [72]. The TGF- α cleavage site is composed of small, hydrophobic residues that would be predicted to be cleaved by an elastase-like protease, and some, but not all, elastase inhibitors were able to prevent cleavage after stimulation with phorbol esters. The inhibitor profile of a partially purified putative TGF- α secretase also indicated an elastase-like activity [67]. Several studies using specific protease inhibitors have implied that TGF- α secretase is a serine protease with an elastase-like specificity. However, recent data have cast doubt upon this supposition [73]. Here it was shown that 3,4-DCI was not able to prevent the release of TGF- α when release was assayed using fluorescence-activated cell sorting (FACS) analysis, as opposed to using specific immunoprecipitation of metabolically labelled protein. Instead, the metalloprotease inhibitor TAPI-2 (Table 3 and Figure 4) was able to inhibit the release of TGF- α from the surface of transfected CHO cells. The explanation for these conflicting data was that the serine-protease inhibitors prevented the trafficking of TGF- α to the cell surface, whereas the metalloprotease inhibitors were able to prevent the cleavage of the ligand from the cell surface.

Tumour necrosis factor- α (TNF- α) ligand/receptor superfamily secretases

TNF- α is a pleiotropic cytokine produced predominantly in response to infection, antigen or injury, and elicits a broad spectrum of biological effects. These effects are mediated by TNF- α binding to cell-surface receptors. In recent years it has become clear that a number of different ligand/receptor pairs

show sequence similarity to TNF- α and TNF receptors. The receptors are Type I and the ligands Type II membrane proteins. The ligand/receptor members of the superfamily play critical roles in immune and inflammatory system regulation (with the exception of the nerve growth factor receptor/ligand pair). With the possible exception of those members of the family for which mRNA splice variants predict that soluble proteins exist, it is likely that all members will be demonstrated to be cleaved from the surface of cells by secretases. Indeed there is also increasing data to suggest that both the ligands and the receptors of many of the members of this family are released by the same, or closely related, secretases [74].

TNF- α secretase

Mature, biologically active human TNF- α is a 17.3 kDa protein that is cleaved from a 26 kDa transmembrane precursor by a secretase [75]. To investigate the processing of the 26 kDa form, site-directed mutagenesis was performed around the cleavage site [76]. Deleting amino acids +1 to +5, -3 to +5, and -3 to -1 (where the cleavage site is at amino acids -1/+1) was unable to prevent the processing of human TNF- α transfected into 3T3 cells, which implies that alternative processing can occur, either through cleavage by other secretases or because TNF- α secretase has a relaxed cleavage site specificity. Only by deleting amino acids +1 to +12 could the cleavage be prevented; anything less than this was ineffective. Similar data were generated using murine TNF- α , where only the +1 to +12 deletion mutant was able to prevent cleavage [77].

There are conflicting data regarding the mechanistic class of TNF- α secretase. There is compelling evidence that synthetic metalloprotease inhibitors (Table 3) are able to prevent the release of TNF- α from THP-1 cells, human monocytes and also in rodent models of endotoxic shock *in vivo* [78–80]. Diastereoisomeric specificity of the hydroxamate metalloprotease inhibitor TAPI was demonstrated in studies *in vitro* [78], thus ruling out non-specific effects due to compound toxicity. The partially purified TNF- α secretase was also able to cleave purified recombinant TNF- α as well as a 20-mer peptide that spanned the TNF- α cleavage site [78]. However, other studies have shown that serine proteases are capable of processing TNF- α [81,82]. It is possible that a cascade of proteolytic events is needed for TNF- α release. Thus, if the metalloprotease activity that has been demonstrated to release TNF- α is present as a proenzyme, as are matrix metalloproteases, then a serine protease may be involved in protease activation.

Fas ligand (FasL) secretase

FasL is a 40 kDa protein expressed on activated T and NK cells that mediates apoptosis via the Fas receptor. Recently, in FasL-transfected COS cells, a 26 kDa soluble form of the protein was detected in the culture supernatant [83]. In addition, the production of soluble FasL was up-regulated by treatment with phorbol ester and ionomycin. Further work has established that the release of FasL into the medium is associated with a reduction in cell-associated full-length FasL, thus establishing a precursor-product relationship [84]. FACS analysis demonstrated that treatment with the matrix-metalloprotease inhibitor BB94 (Figure 4) resulted in an increase in the amount of cell-surface FasL, together with a reduction in the production of soluble FasL. A range of hydroxamate metalloprotease inhibitors was able, in a dose-dependent manner, to prevent the release of FasL after phorbol ester and ionomycin treatment [84].

Tumour necrosis factor receptor (TNFR) secretase

The actions of TNF- α are mediated via a 55/60 kDa receptor (TNFR-I) and a 75/80 kDa receptor (TNFR-II). Early evidence that the TNFRs may exist in soluble forms came from the observation that TNF- α -binding proteins could be purified from urine [85]. Studies using cells transfected with TNFR-I demonstrated that the soluble TNFR-binding protein was derived from the full-length membrane-associated form [86], and C-terminal sequencing of the soluble form revealed the likely cleavage point to be at Asn¹⁷²-Val¹⁷³ [87], which was ten amino acids distal from the transmembrane domain. Site-directed mutagenesis of the cleavage site revealed that the Asn¹⁷² \rightarrow Gly, Val¹⁷³ \rightarrow Ala double mutation was able to prevent phorbol ester-stimulated and constitutive release.

The features that govern secretase-mediated release of TNFR-I were thoroughly investigated by using an extensive range of deletion, domain replacement and Ala substitution mutants made to the juxtamembrane stalk region between the transmembrane domain and the cysteine-rich extracellular domain [88]. Replacement of the stalk region of TNFR-I with the equivalent region from the epidermal growth factor receptor (EGFR) (which is not subject to secretase-mediated release) was able to prevent cleavage, and, conversely, introduction of the TNFR-I stalk region into the EGFR was sufficient to mediate release. Deletion of the stalk region completely, deletion of the ten amino acids from around the cleavage site (Ile¹⁷⁰-Ser¹⁷⁹), and a five-amino-acid deletion from either Ile¹⁷⁰ to Lys¹⁷⁴ or from Gly¹⁷⁵ to Ser¹⁷⁹ were all able to prevent phorbol ester-stimulated release of TNFR-I, whereas a three-amino-acid deletion from Glu¹⁷⁷ to Ser¹⁷⁹ decreased the shedding to some extent. Overlapping two-amino-acid deletions from Glu¹⁷¹ to Gly¹⁸⁰ revealed that only those deletions involving the P₁/P₁' amino acids were able to prevent cleavage, with deletion of the P₁' Val¹⁷³ being the more effective. Changing Val¹⁷³ to Asp, Gly or Pro prevented cleavage, whereas Ala 'panning' (where each amino acid from Glu¹⁷¹ to Thr¹⁸¹ was changed to Ala in turn) failed to prevent release of TNFR-I. This study revealed some interesting features regarding the nature of the secretase-mediated cleavage. From the deletion mutants it would seem that three, but not five or more, amino acids can be removed from the 16-amino-acid stalk region without preventing cleavage. This implies that, for TNFR-I, there is a minimum of a 13-amino-acid stalk region required, which could be interpreted as being sufficient to allow access and binding of the secretase to the cleavage site (see Figure 2b). The secretase does have cleavage-sequence specificity, as deletion of the P₁/P₁' residues prevents cleavage; in addition, the P₁' residue appears to be dominant in determining specificity, which is similar to the matrix metalloproteases. Also, amino acid changes predicted to disrupt the conformation of the stalk region are effective inhibitors of secretase action.

Recent data have demonstrated that phorbol ester-stimulated TNFR-I release from various cells and cell lines can be inhibited by the inhibitor TAPI (Figure 4 and Table 3) [89]. An extensive range of other class-specific protease inhibitors were shown to be ineffective, including tissue inhibitor of metalloproteases 1 (TIMP1) and TIMP2. This would suggest that the protease is not a member of the matrix-metalloprotease family. Similarly, TNFR-II is proteolytically cleaved from cells, and the release can also be inhibited using TAPI (Table 3) [90]. The cleavage site for TNFR-II is Val¹⁹²-His¹⁹³ [88], which is 14 amino acids from the cysteine-rich extracellular domain, compared with six amino acids for TNFR-I. The stalk region is much longer in TNFR-II compared with TNFR-I, resulting in the cleavage site being 43 amino acids distant from the transmembrane domain region

(Table 2). Although both TNFR-I and TNFR-II receptors have cysteine-rich extracellular domains that probably form closely folded globular structures, their respective stalk regions are of different lengths. Whereas there would appear to be a minimum sequence length for the stalk, below which cleavage does not occur, when the stalk region is elongated, cleavage occurs distal to the transmembrane domain but proximal to the released globular domain. Thus there would appear not to be a distance restriction conferred by the protease, but more a restriction, based on preventing access, imposed by the tertiary structure of the substrate (in this case, a receptor) (see Figure 2b). A recent study has described an assay using cells expressing TNFR-II at the cell surface to partially characterize a soluble metallosecretase that is itself secreted into the media upon phorbol ester stimulation of the cells [91].

CD30 secretase

CD30 is a 120 kDa protein that was originally defined as an activation marker associated with malignant lymphoma cells from patients with Hodgkin's disease. The sera of Hodgkin's-disease patients contain soluble CD30, and recent studies have confirmed that soluble CD30 is released from the surface of cells by a metalloprotease. BB2116 (Figure 4) was able to preserve surface CD30 (as assayed using FACS analysis) after phorbol ester stimulation (Table 3), whereas a range of inhibitors against other classes of protease were inactive [92]. Iodoacetamide, an alkylating agent, was able to upregulate the cleavage of CD30. This finding is reminiscent of the matrix metalloproteases, where alkylation of the cysteine involved in the cysteine switch mechanism converts the enzyme from the latent to the active form [93].

KL secretase

KL (stem-cell factor; steel factor; mast-cell growth factor) is a pleiotropic growth factor involved in haemopoiesis. The cognate receptor for KL is *C-kit* which has tyrosine kinase activity. The KL gene is alternatively spliced, giving rise to two major isoforms, known as KL-1 and KL-2 [94]. Both murine KL-1 and KL-2 are Type I integral membrane proteins of 248 and 220 amino acids, respectively, with a 36-amino-acid cytoplasmic tail. A precursor-product relationship between the membrane-associated and the soluble form of the ligand was demonstrated using metabolic labelling of proteins followed by specific immunoprecipitation [95]. These studies also confirmed that release of the soluble form of KL-1 and KL-2 could be up-regulated with phorbol esters as well as calcium ionophore treatment. The cleavage site for KL-1 was defined from purified soluble KL as being either at Ala¹⁶⁵-Ala¹⁶⁶ or at Ala¹⁶⁶-Ser¹⁶⁷ (Table 2) [96]. The KL-1 cleavage site is encoded by exon 6, which is spliced out in KL-2. Consequently, KL-2 is the predominant membrane-associated form of the ligand, although it is subject to some secretase cleavage. The amino acid specificity of KL secretase was investigated using site-directed mutagenesis within the cleavage site [97]. It was shown that changing the Ala-Ala sequence and removing the flanking residues, or deleting the Val-Ala-Ala-Ser cleavage site of KL-1, was ineffective in preventing cleavage of the protein from the membrane, although the rate of release was lower. From the sizes of the released KL-1 proteins, it appeared that release was occurring at an alternative site nearer to the transmembrane domain. Further deletion of a region with small hydrophobic residues (Lys¹⁷⁸-Ala-Ala-Lys¹⁸¹) completely prevented release of KL-1, strongly implicating this region as the alternative cleavage site. This putative cleavage site

is present in KL-2, and is most probably, therefore, the site at which KL-2 cleavage occurs.

The cytoplasmic domain is not required for appropriate secretase cleavage of KL-1, although membrane anchorage is a prerequisite [69]. The general parameters that govern secretase cleavage of KL-1 were investigated by inserting two exon 6 regions, and therefore two identical cleavage regions, in tandem into KL-1. In the resultant KL-1 mutant proteins, only the cleavage region proximal to the membrane was utilized by the secretase, implying KL-1 secretase was subject to some form of distance restriction. An extensive protease-inhibitor profile of KL-1 and KL-2 [72] has demonstrated that KL processing is prevented by a very similar collection of reagents as is TGF- α , with some subtle differences between KL-1 and KL-2. The release of both ligands was inhibited by a number of chymotrypsin inhibitors and by an elastase inhibitor. The release of KL-1 was additionally inhibited by another elastase inhibitor and by the serine-protease inhibitors DFP and 3,4-DCI. General metallo-, cysteine- and aspartic-protease inhibitors were without effect on either ligand. Thus KL secretase would appear to be similar in many respects to TGF- α secretase. However, given recent data regarding the activity of metalloprotease inhibitors [73], it may be that the serine-protease inhibitors are inhibiting biochemical events, such as trafficking, that lead eventually to cleavage by a metalloprotease.

Interleukin 6 receptor (IL6R) secretase

Interleukin 6 is involved in the regulation of the immune response, haematopoiesis and the acute-phase response. IL6R is a 468-amino-acid Type I integral membrane protein [98] that forms a functional receptor when complexed with gp130, the tyrosine kinase signal-transducing protein. IL6R is a member of the haematopoietic receptor family characterized by conserved cysteine residues and a Trp-Ser-Xaa-Trp-Ser motif in the extracellular domain. A precursor-product relationship was demonstrated between the membrane-associated and the released soluble form of IL6R using metabolic labelling and specific immunoprecipitation with COS-7 cells transfected with IL6R [99]. The cleavage of IL6R could be up-regulated with phorbol ester treatment and by co-transfecting the cells with PKC.

A very thorough investigation of the parameters that regulate the IL6R secretase [100] identified the cleavage site to be at Gln³⁵⁷-Asp³⁵⁸. This would place the cleavage position just a single amino acid away from the transmembrane domain, which seems unusual on the basis of data on other secretase-cleaved proteins. However, as the predicted transmembrane domain includes 28 amino acids, rather more than is normally required, it could well be that there is a greater distance between the cleavage site and the membrane surface. Two deletions of four and five amino acids either side of the cleavage site reduced, but did not abolish, the cleavage of IL6R, implying that alternative cleavage sites could be used. A ten-amino-acid deletion mutant centred on the scissile bond effectively abolished cleavage. A range of point mutations to the P₁ Gln residue and the P₁' Asp residue were not able to prevent cleavage completely, although mutations made to the P₁' residue tended to be more effective in this regard. The nature of IL6R secretase was further illuminated when it was demonstrated that the metalloprotease inhibitor TAPI was able to prevent the release of the receptor from transfected COS cells and from THP-1 cells (Table 3) [89]. It was shown that this was a direct effect of the inhibitor on the secretase, rather than on other aspects of cellular metabolism, by demonstrating efficacy in the presence of cycloheximide to

prevent *de novo* protein synthesis. TAPI was able to inhibit both the basal and phorbol ester-stimulated release of IL6R.

CD43, CD44 and CD16 secretase(s)

CD43 (sialophorin, the major leucocyte sialoglycoprotein), CD44 (hyaluronate receptor) and the GPI-anchored CD16-I (low-affinity Fc γ receptor) are all enzymically cleaved from the surface of stimulated leucocytes. The release of all three proteins was inhibited by either the metalloprotease inhibitor 1,10-phenanthroline or the serine-protease inhibitors *N*²-*p*-tosyl-L-lysylchloromethane ('TLCK') and 3,4-DCI, but not by low-molecular-mass matrix-metalloprotease inhibitors [101,102], leading those authors to suggest that there is an enzymic cascade consisting of a metalloprotease and a serine protease. The proteolytic release of CD43 and CD44 was stimulated by treatment of human neutrophils with TNF- α , *N*-formyl-L-Met-L-Leu-L-Phe or phorbol esters [103,104], and cross-linking of the proteins with monoclonal antibodies [102]. The transmembrane polypeptide anchored form of CD16-II is also spontaneously released from the surface of certain cells by a phorbol ester-activated process [105]. This release was not blocked by serine- or aspartic-protease inhibitors, was partly blocked by EDTA and EGTA, and completely inhibited by 1,10-phenanthroline, with the inhibition being reversed by Zn²⁺.

L-Selectin secretase

L-selectin (human Leu-8; murine MEL-14; LAM-1 antigen) is rapidly released from the plasma membrane by proteolytic cleavage upon neutrophil activation [106,107]. The release of this protein is stimulated by phorbol ester or TNF- α treatment of cells [103,108]. The site of cleavage within L-selectin has been identified at Lys³²¹-Ser³²², 11 residues distal to the membrane-spanning domain [109]. Replacing the cleavage site in L-selectin with the corresponding sequence of E-selectin, which is not secreted from the cell surface, prevented secretion of L-selectin [110]. Point mutations at the cleavage site, as well as mutations of multiple conserved amino acids within the cleavage domain, did not significantly affect the release of L-selectin, although deletions of four or five amino acids in the cleavage domain did inhibit secretion. One deletion mutant that retained the native cleavage site was not cleaved, although replacing the deleted residues with five Ala residues restored cleavage [110]. A similar study also showed that the length of the membrane-proximal region was critical, since truncations of this region completely abolished cleavage [111]. These results led both groups to suggest that the L-selectin secretase has a relaxed sequence specificity and cleaves the receptor at a specific distance from the membrane, and that cleavage depends on the physical length or other secondary- or tertiary-structural characteristics of the cleavage domain. Another interpretation of these data is that the secretase requires a minimum stalk length in order to gain access to the cleavage site (see Figure 2b).

The secretase involved in the release of L-selectin does not appear to be inhibited by serine-, metallo-, aspartic- or thiol-protease inhibitors [102,109]. Recently, however, it has been shown that a hydroxamic acid-based metalloprotease inhibitor KD-IX-73-4 (Figure 4), blocks the release of both wild-type L-selectin and an L-selectin-alkaline phosphatase reporter construct (Table 3) [112]. The diastereoisomer was 20–25-fold less potent. In addition, KD-IX-73-4 reduces neutrophil rolling velocity under hydrodynamic flow, an event that is mediated, at least in part, by L-selectin [113]. Other metalloprotease inhibitors, Ro 31-9790 and TAPI-2 (Figure 4), have also been shown recently to block the release of L-selectin from leucocytes,

neutrophils, eosinophils and lymphocytes [114,115]. Although Ro 31-9790 was designed as a matrix-metalloprotease inhibitor and L-selectin was susceptible to cleavage by recombinant fibroblast collagenase, TIMP was without inhibitory effect, and lymphocytes secreting L-selectin had no detectable collagenase activity at the cell surface [115], indicating that L-selectin secretase is distinct from the matrix metalloproteases.

Folate receptor secretase

The mammalian folate receptor has been identified as a GPI-anchored protein [116,117], although there appear to be multiple genes encoding this membrane protein, which may give rise to alternatively anchored forms. Thus, as well as being acted on by GPI-specific phospholipases, there is evidence indicating that the folate receptor in human placenta and a human nasopharyngeal carcinoma cell line is cleaved by a membrane-associated metalloprotease [118–120]. Interestingly, like the ACE secretase [50], the folate receptor secretase was not inhibited by EGTA, and the inhibition by EDTA could be reversed by Mg²⁺. The site of cleavage within the folate receptor has been identified as lying in the region between Ala²²⁶ and Tyr²²⁹ [119].

Recently, a metalloprotease that cleaves the folate receptor from the membrane has been purified from human placental membranes following solubilization with Triton X-114 [121]. The purification procedure involved only two chromatographic steps, chromatography on concanavalin A-Sepharose and reverse-phase HPLC, with the activity being monitored using mature ¹²⁵I-labelled hydrophobic placental folate receptor as the substrate. The purified protease appeared as a single band, on SDS/PAGE, of 63 kDa, a value that was decreased to 58 kDa following removal of the N-linked sugars. The partially purified enzyme was inhibited by EDTA and activated by Ca²⁺, Mg²⁺, Mn²⁺ and Zn²⁺. The effect of more selective metalloprotease inhibitors on this activity, or its ability to cleave other membrane proteins, has yet to be determined. If substantiated, this represents the first purification of a membrane protein secretase.

SECRETASE ASSAYS

Whole-cell assays

The majority of studies on membrane protein secretases have employed whole-cell systems utilizing either natural or recombinant cell lines that express both the membrane protein and its secretase. The activity of the secretase is usually detected and quantified by monitoring the presence of the cleaved, hydrophilic form of the membrane protein in the cell medium. The advantages of such systems are that the media containing the solubilized protein can readily be separated from the membrane-bound form on the cells, and the effect of various agents (e.g. phorbol esters, transport inhibitors, etc) on the activity of the secretase can be studied. The released form of the protein can be detected by activity measurements or with specific antibodies, often following metabolic labelling with [³⁵S]Met or surface labelling (biotinylation or iodination). Alternatively, the disappearance of the membrane-bound form can be followed by, for example, flow cytometry. The major disadvantages of these whole-cell assays comes when screening for potential inhibitors of the secretase, where it is difficult to distinguish between a direct inhibitory effect on the secretase and other aspects of cell metabolism or viability. This has been highlighted recently with the observation that although the release of TGF- α , L-selectin, IL6R and APP from the surface of CHO cells can be blocked by both metalloprotease inhibitors (TAPI-2 and 1,10-phenanthroline) and serine-protease inhibitors, the latter may do so by interfering with the

maturation and transport of the proteins to the cell surface [73]. In addition, such whole-cell assays cannot be used for monitoring the purification of the secretase.

Cell-free assays

Only a few studies have utilized cell-free assays for membrane protein secretases ([2,31,50,51,113]; S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work). The most direct cell-free assay involves isolation of a membrane fraction in which both the membrane protein and its secretase are present, and then to use this preparation to study the action and properties of the secretase [2,50]. Isolation of relatively pure plasma-membrane fractions enables the action of the secretase to be localized to the cell surface, and either natural tissue sources or recombinant cell lines can be employed. Such 'co-localized' assay systems can be readily used to characterize the structural and catalytic/inhibitor properties of the secretase, although care has to be taken that it is the action of the secretase that is being studied and not the action of another non-specific protease. Again, such co-localized assay systems are not suitable for monitoring the purification of the secretase, as the initial solubilization step will disrupt the membrane structure, destroying the co-localized system.

Following detergent solubilization of ACE secretase from porcine kidney membranes, we attempted to measure the activity of the secretase by adding back a source of purified amphipathic ACE to act as substrate. Although this form of ACE contains the secretase cleavage site, no proteolytic cleavage was observed (S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work). Following the observation that porcine intestinal brush-border membranes contained ACE but lacked the secretase [50], we employed these membranes as a source of the substrate in order to ascertain whether there was some other factor in the membranes that was required to regain the secretase activity. With the intestinal membranes as the source of the substrate, ACE, we were now able to detect EDTA-sensitive secretase activity in the solubilized secretase preparation (S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work). A more refined system involves incorporation of the purified amphipathic form of ACE into artificial lipid vesicles (S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work). Trypsin-solubilized secretase, which cannot reinsert into the lipid vesicles, also functions in this assay, implying that it is the substrate (ACE) that needs to be in the membrane. This observation, whereby membrane protein secretases require their substrates to be membrane-inserted in order to act, may be a general feature of such enzymes, as a similar phenomenon has been observed recently for the α - and β -secretases that process APP [31,38] and the metallosecretase that cleaves KL-1 [69].

Recently, cell-free assays have been described for the metallosecretases responsible for the release of L-selectin [113] and the folate receptor [121]. Purified L-selectin containing a C-terminal FLAG epitope (Kodak) was immobilized on agarose beads by concanavalin A. A Triton X-100-solubilized cell membrane preparation was then incubated with the L-selectin-agarose slurry and generation of the C-terminal 6 kDa cleavage fragment detected with an anti-FLAG monoclonal antibody by immunoelectrophoretic blot analysis. In the case of the folate receptor, again full-length protein substrate was used to monitor the activity of its secretase (see above). All these studies indicate that only the full-length protein, in some cases membrane-bound, can

serve as substrate for the relevant secretase. In the past, numerous studies have attempted to use short synthetic peptide substrates based on the sequence around the cleavage site. To our knowledge, none of the activities identified using such substrates have been shown to cleave the full-length protein in an appropriate fashion. For example, a recent study clearly showed that although cathepsin G and endopeptidase-24.15 (thimet oligopeptidase; EC 3.4.24.15) could cleave a short peptide based on the APP β -secretase cleavage site, neither protease was capable of cleaving full-length wild-type APP [122].

Methods for distinguishing between the amphipathic and hydrophilic forms of a membrane protein

To assay for the activity of a membrane protein secretase it is essential to differentiate the released form of the protein (the product of the reaction) from the membrane-bound form (the substrate). A major difference between the released, hydrophilic form and the membrane-bound, amphipathic form of such membrane proteins is the absence or presence, respectively, of the hydrophobic membrane-anchoring domain. The hydrophilic and amphipathic forms of a membrane protein can be distinguished by their difference in size on SDS/PAGE. However, since cleavage usually occurs close to the membrane surface, releasing the bulk of the protein from the small membrane-spanning and cytosolic domains, the size difference between the two forms may be relatively small, thus making it difficult to distinguish between them (see, for example, [2]). Because most plasma-membrane proteins are often glycosylated extensively and heterogeneously, resulting in broad bands on SDS/PAGE, the difference in size between the hydrophilic and amphipathic forms of a protein can be made more apparent if the protein is first deglycosylated [51]. Obviously, care has to be taken that the proteins have been fully deglycosylated every time, and the expense and time involved do not lend this method to being useful for multiple assays. A further refinement of the separation of the hydrophilic and amphipathic forms of a protein on SDS/PAGE is to perform immunoelectrophoretic blot analysis with site-specific antibodies which selectively recognize regions of the protein either side of the secretase cleavage site [61]. Thus, although there may be no discernible difference in size between the two forms, an antibody raised to the cytosolic domain will cross-react only with the full-length amphipathic form. Alternatively, antibodies can be generated that recognize the new N- or C-terminus exposed on cleavage by the secretase. Such neo-epitope antibodies have been used primarily in studies on the cleavage of APP by β -secretase [123].

Alternative techniques to distinguish between the amphipathic and hydrophilic forms of a membrane protein directly exploit the hydrophobic property of the membrane-anchoring domain (reviewed in [124]). One of the most definitive methods for determining whether the form of a protein under investigation has the hydrophobic anchoring domain is reconstitution into artificial lipid vesicles [2]. However, such reconstitution experiments are time-consuming and not useful as a routine procedure. Probably the most sensitive, rapid and convenient method for separating the hydrophilic and amphipathic forms of a protein is temperature-induced phase separation in Triton X-114 [124,125]. This method is rapid and convenient to use when screening multiple samples, factors which have led us ([50]; S. Parvathy, S. Y. Oppong, E. Karran, D. R. Buckle, A. J. Turner and N. Hooper, unpublished work) and others to use it routinely for separation of the hydrophilic and amphipathic forms of a membrane protein when assaying for its secretase.

CONCLUSIONS AND FUTURE PERSPECTIVES

From this Review some general features of the membrane protein secretases are apparent. It is clear that intrinsically they have been exceptionally difficult to characterize at the biochemical and molecular level, and, to date, not a single cDNA encoding a secretase has been published. Most probably this is because traditional approaches to purifying and assaying for proteases are inappropriate for secretases, where the use of whole protein substrates, as opposed to short peptides, and in some cases membrane insertion of the substrate, would seem to be prerequisites for proteolytic activity. There is the common observation that phorbol ester treatment up-regulates secretase cleavage. However, currently it is not known whether this represents an increase in the activity of the secretase, increased production of the substrate or increased trafficking of the substrate to the site of secretase action. Most of the secretases appear to be located and act either at the cell surface, or in exo- or endo-cytic vesicles close to the plasma membrane.

The majority of secretases are metalloproteases, with a number of them inhibited by TAPI or other structurally related hydroxamic acid-based compounds (Figure 4 and Table 3). From the lack of inhibition of TIMP on secretase-mediated events, it would seem unlikely that secretases are closely related members of the matrix-metalloprotease family [89]. The observation that the release of some membrane proteins is blocked by both metallo- and serine-protease inhibitors has led to the suggestion that a proteolytic cascade is involved. However, recent data indicate that serine-protease inhibitors may affect the trafficking of substrate proteins to the site of secretase action rather than the release process itself [73]. The β - and γ -secretases involved in APP processing seem to be atypical, insofar as their activities are not up-regulated by phorbol esters, nor is there any evidence that they are metalloproteases. In contrast, the α -secretase activity would appear to be a metallosecretase.

Although early studies suggested that secretase action was limited to a particular distance from the membrane, this concept has not been definitely established. Is cleavage limited because the secretase active site is tethered close to the membrane, or is it that the substrate restricts access of the secretase by steric hindrance? Although cleavage is likely to be governed by a range of factors, including the amino acid sequence at and around the cleavage site, we suggest that the latter scenario is more likely. Of interest in this regard is the cleavage of TNFR-I and TNFR-II, which have stalk regions of 16 and 56 amino acids respectively [88]. Here the cleavage occurs at roughly the same distance from the extracellular receptor domain, rather than from the membrane, suggesting that the secretase is able to cleave at a range of distances from the membrane. A similar conclusion has also recently been drawn from studies on stalk-region mutants of ACE, where it was shown that the secretase requires an accessible stalk and positions itself primarily with respect to the proximal extracellular domain [126].

The topology of the proteins being cleaved by the secretases is also an intriguing aspect. In the same cells, the cleavage of both Type I and Type II proteins can be up-regulated by phorbol esters and then inhibited with very similar potency by the same metalloprotease inhibitor [89]. However, the peptide backbone of the substrate runs in opposite directions relative to the membrane. Thus, for the same secretase to cleave both Type I and II proteins, some spatial accommodation of the proteins will be required to allow the appropriate orientation of peptide backbone into the active site (assuming that the secretase is itself membrane-associated). As some secretase cleavages occur in fairly short stalk regions (e.g. TGF- α), where hindrance caused

by the extracellular portion of the protein may preclude the stalk from adopting a different orientation, it seems more likely that the secretase is the flexible partner. Another possibility is that two subfamilies of secretases exist, with similar catalytic domains within Type I and Type II proteins that only cleave their topologically cognate proteins.

Despite our lack of knowledge in this area, it seems clear that there are elements within the secretase biochemical pathway that are shared by most cells. Thus, transfection of Cos or CHO cells with cDNAs encoding TGF- α , KL, ACE, L-selectin or the IL6-R results in the production of soluble proteins. Also, the observation that mutant CHO cells were unable to cleave both TGF- α and APP [71] and that the metalloprotease inhibitor TAPI-2 blocked the release of TGF- α , L-selectin, IL6-R and APP from these cells [73], further supports this hypothesis. Of course, it is likely that these metallo-inhibitors are relatively non-specific inhibitors of several related secretases (cf. inhibition of the thermolysin, endopeptidase-24.11 and endothelin converting enzyme by phosphoramidon [127], and inhibition of several mammalian aminopeptidases by amastatin [128]). Ultimately, the question of the range and complexity of these proteases will only be answered following the purification and cDNA cloning of one or more secretases.

Given the wide diversity of proteins that are secretase substrates, it is clear that inhibition of some of the secretases could offer novel therapeutic targets for certain diseases. Certainly, considerable effort is being expended to find inhibitors of the APP β -secretase and γ -secretase with a view to reducing amyloid burden as a treatment for Alzheimer's disease [43]. Also, inhibitors of the secretases that release TNF- α may offer relief in autoimmune diseases such as arthritis [78–80]. The challenge facing the pharmaceutical industry is to design specific inhibitors that target discrete secretases. To emphasize this point, a poorly selective secretase inhibitor may prevent cleavage of both a ligand and its cognate signalling receptor, with little overall change to the relevant physiological response [74]. Potent, selective secretase inhibitors will only be developed by medicinal chemists when, at the very minimum, cell-free secretase assay systems have been configured. More selective inhibitor specificity will only be derived using purified secretase assay systems, optimally together with knowledge of the crystal structure of the active site. Given that secretases are integral membrane proteins, this remains a formidable task.

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